Sequencing of plasmids pAMBL1 and pAMBL2 from Pseudomonas aeruginosa reveals a blaVIM-1amplification causing high-level carbapenem resistance

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Abstract: BACKGROUND: Carbapenemases are a major concern for the treatment of infectious diseases caused by Gram-negative bacteria. Although plasmids are responsible for the spread of resistance genes among these pathogens, there is limited information on the nature of the mobile genetic elements carrying carbapenemases in Pseudomonas aeruginosa. METHODS: We combined data from two different next-generation sequencing platforms, Illumina HiSeq2000 and PacBio RSII, to obtain the complete nucleotide sequences of two blaVIM-1-carrying plasmids (pAMBL1 and pAMBL2) isolated from P. aeruginosa clinical isolates. RESULTS: Plasmid pAMBL1 has 26 440 bp and carries a RepA_C family replication protein. pAMBL1 is similar to plasmids pNOR-2000 and pKLC102 from P. aeruginosa and pAX22 from Achromobacter xylosoxidans, which also carry VIM-type carbapenemases. pAMBL2 is a 24 133 bp plasmid with a replication protein that belongs to the $Rep_3 family. It shows a high degree of homology with a fragment of the bla VIM-1-bearing plasmid pPC9 from Pseudomonas putida. Plasmid pAMBL2 carries three copies of the bla VIM-1 cassette in an In70 class 1 integron conferring, unlike pAMBL1, high-level resistance to carbapenems. CONCLUSIONS We present two new plasmids coding for VIM-1 carbapenemase from P. aeruginosa and report that the presence of three copie 1 in pAMBL2 produces high-level resistance to carbapenems.$

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Sequencing of plasmids pAMBL1 and pAMBL2 from *Pseudomonas aeruginosa* reveals a *bla*$_{VIM-1}$ amplification causing high-level carbapenem resistance

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Running title: Characterization of plasmids pAMBL1 and pAMBL2.

Keywords: Plasmid-mediated resistance, VIM, carbapenemase, antibiotic multiresistance, *Pseudomonas aeruginosa*, gene amplification.

Synopsis

Objectives: Carbapenemases are a major concern for the treatment of infectious diseases produced by Gram-negative bacteria. Although plasmids are responsible for the spread of resistance genes among these pathogens, there is limited information on the nature of the mobile genetic elements carrying carbapenemases in *Pseudomonas aeruginosa.*

Methods: We combined data from two different next generation sequencing platforms, Ilumina HiSeq2000 and PacBio RSII, to obtain the complete nucleotide sequence of two bla_{VIM-1}-carrying plasmids (pAMBL1 and pAMBL2) isolated from *P. aeruginosa* clinical isolates.

Results: Plasmid pAMBL1 has 26,440 bp and carries a RepA_C family replication protein. pAMBL1 is similar to plasmids pNOR-2000 and pKLC102 from *P. aeruginosa* and pAX22 from *Achromobacter xylosoxidans,* which also carry VIM-type carbapenemases. pAMBL2 is a 24,133 bp plasmid with a replication protein that belongs to the Rep_3 family. It shows high degree of homology with a fragment of the bla_{VIM-1}-bearing plasmid pPC9 from *Pseudomonas putida*. Plasmid pAMBL2 carries three copies of the bla_{VIM-1} cassette in an In70 class 1 integron conferring, unlike pAMBL1, high level resistance to carbapenems.

Conclusions: Here we present two new plasmids coding for VIM-1 carbapenemase from *P. aeruginosa* and we report that the presence of three copies of bla_{VIM-1} in pAMBL2 produces high-level resistance to carbapenems.

Introduction

P. aeruginosa produces opportunistic infections that are hard to treat due to its great ability to develop resistance to antibiotics. Although chromosomally-encoded resistance mechanisms are often responsible for this phenotype,[1] plasmids also play a key role in the acquisition of antibiotic multi-drug-resistance in this species. Plasmids from *P. aeruginosa* often carry the same antibiotic resistance genes found on mobile genetic elements from Enterobacteriaceae. Among these genes, those encoding metallo-β-lactamases such as VIM-type enzymes are specially important since they can hydrolyse all β-lactams including carbapenems, one of the last resorts of therapeutic treatment for infections caused by Enterobacteriaceae. Despite their clinical significance, there is little information available on the nature of plasmids carrying carbapenemases in *P. aeruginosa*,[2] or on the routes of dissemination of these resistance genes between pseudomonas and enterobacteria.

pAMBL1 and pAMBL2 are two $bla_{\text{VIM-1}}$-carrying plasmids recovered from *P. aeruginosa* clinical isolates at the *Ramon y Cajal University Hospital* in Madrid, Spain, in 2006-2007, during an outbreak of VIM-producing bacteria.[3] Here, we combined Ilumina HiSeq2000 and PacBio RSII technologies to determine the complete nucleotide sequence of pAMBL1 and pAMBL2. This strategy allowed us to detect three copies of $bla_{\text{VIM-1}}$ β-lactamase gene inside an integron in pAMBL2, which conferred high-level resistance to meropenem in *P. aeruginosa* PAO1.

Materials and methods

Bacterial strains, culture conditions and antibiotic susceptibility testing.

Plasmid pAMBL1 and pAMBL2 were recovered from *P. aeruginosa* isolates in the pneumology ward in 2006 and in the urology ward in 2007, respectively, at the *Ramon y Cajal University Hospital* in Madrid.[3] Bacterial strains were cultured in LB broth at 37°C (225 rpm) and on Luria Bertani agar plates at 37°C (Fisher Scientific, USA). Plasmids were electroporated into *P. aeruginosa* PAO1.[4] Transformants were selected on plates with carbenicillin 256 mg/L and ceftazidime 50 mg/L. We performed conjugation experiments as previously described[3] using a GFP-tagged *P. aeruginosa* PAO1 (chloramphenicol and gentamicin resistant)[4] as a recipient strain and PAO1/pAMBL1 and PAO1/pAMBL2 as donors. Susceptibility testing was performed by broth microdilution method according to the CLSI guidelines.[5] The MICs were determined as the mean of three or four independent experiments (Table 1).

DNA analysis, sequencing and bioinformatics

DNA samples were obtained using Qiagen Dneasy Blood and Tissue Kit (Qiagen, USA) and quantified using QuantiFluor dsDNA system (Promega, USA). Library preparation and sequencing was performed at the Wellcome Trust Centre for Human Genetics, University of Oxford (using HiSeq2000 and 100-bp-paired end reads, average median depth of coverage 70x) and at The Genome Analysis Centre in Norwich (PacBio RS platform using P5-C3 chemistry, average median depth of coverage 50x). To assemble de novo the paired end reads obtained with Illumina technology we used A5 pipeline (developed for microbial genomes).[6] We used Geneious 7.1.7 (Biomatters, USA) to analyse the reads from PacBio. To annotate the plasmids we used RAST server,[7] and confirmed the annotation doing BLASTP similarity searches of each predicted protein. Plasmid sequences

were confirmed by restriction profile with endonucleases *Hind*III, *EcoR*I, *Xho*I, *Kpn*I, *BamH*I (New England BioLabs, USA), by PCR mapping and by Sanger sequencing the complete integron in pAMBL2 and all the ambiguous residues (Table S1). RNA extraction and RNA-Seq analysis were performed as described in San Millan *et al.* 2015.[8]

Results and discussion

Sequencing of plasmids pAMBL1 and pAMBL2

We transformed *P. aeruginosa* PAO1 with plasmid pAMBL1 and pAMBL2.[4] To determine the complete nucleotide sequence of the plasmids, we extracted the genomic DNA from PAO1/pAMBL1 and PAO1/pAMBL2 and sequenced the samples using Ilumina HiSeq2000. After filtering PAO1 chromosomal DNA and assembling the remaining reads, we obtained a single contig for pAMBL1 that was manually closed to a circle. However, for pAMBL2 we obtained two non-overlapping contigs, which could not be linked by PCR. A limitation of assembling bacterial genomes using Illumina technology is that reads are short (ca. 100bp), and this affects the power to assemble DNA regions containing long repeats and duplications.[9] Therefore, we sequenced both DNA samples using PacBio RSII technology, which has a higher error rate than the Ilumina technology but produces longer reads (average= 6kb). PacBio data helped solving the overall structure of the plasmids. By combining the results obtained with the two platforms, we were able to determine the exact nucleotide sequence of plasmids pAMBL1 and pAMBL2.

This work enlarges the list of plasmids from *P. aeruginosa*, which despite the relevance of this species as opportunistic pathogen, are underrepresented in the plasmid genome database (http://www.ncbi.nlm.nih.gov/genome/?term=pseudomonas+aeruginosa).

Analysis of pAMBL1

pAMBL1 conferred resistance to gentamicin, carbenicillin, ceftazidime, meropenem and mercury in *P. aeruginosa* PAO1 (Table 1) and was not able to conjugate under our experimental conditions. It was 26,440 bp in size, had an average GC content of 63.5% and contained 29 ORFs (Figure 1a). This plasmid carried a replication protein belonging to the RepA_C family and presented a backbone structure containing the replication, partitioning and mobilization genes which was very similar to the equivalent region in three plasmids: (i) the bla_{VIM-2} carrying plasmid pNOR-2000 from *P. aeruginosa*,[10] (ii) plasmid pAX22 from *Achromobacter xylosoxidans*, which also carries bla_{VIM-1},[11] and (iii) Tn*CP23*, a plasmid inserted into the chromosomally integrated element pKLC102 of *P. aeruginosa* strain C[12] (Figure 1a). pAMBL1 also carried a Tn*5563a* element,[13] inserted in the *traG* gene, carrying putative mercury resistance genes (Table 1), and a defective Tn*402* transposon carrying *aadB* and bla_{VIM-1} cassettes in a class 1 integron. Additionally, pAMBL1 also harboured a serine protease gene, *degP*, similar to the one in pNOR-2000,[10] and a putative efflux pump and its transcriptional regulator showing 99% nucleotide identity with those found in *Enterobacter* spp. plasmid pENT01 (Accession NC_021492.1).

Analysis of pAMBL2

Plasmid pAMBL2 conferred resistance to streptomycin, kanamycin, carbenicillin, ceftazidime and meropenem in PAO1 (Table 1) and it was unable to conjugate. This plasmid presented a length of 24,133 bp, with an average GC content of 60.4%, and it coded for 35 predicted ORFs including a Rep_3 family replication protein. pAMBL2 was very similar to a segment of pPC9, an 80 kb multi-drug resistance plasmid from a clinical isolate of *Pseudomonas putida*[14]. pAMBL2 backbone was also similar to pCT14 from a *Pseudomonas* spp. isolated from activated sludge (Figure 1b).[15] Apart from the replication and partitioning genes, pAMBL2 also carried an IS*Pa*17 element coding for a toxin-antitoxin system.[16] IS*Pa*17 has been suggested to mobilize defective Tn*402*-like transposons carrying class 1 integrons.[11] We found that pAMBL2 contained three copies of the $bla_{\text{VIM-1}}$ carbapenemase gene cassette inside the In70 class 1 integron, which also carried *aacA4*, *aadA1*, *qacEΔ1* and *sul1* cassettes.

Interestingly, pAMBL2 conferred high-level resistance to meropenem in *P. aeruginosa* PAO1 (MIC, average= 192 mg/L, SD=74 mg/L, n= 4). Actually, meropenem resistance was higher in PAO1/pAMBL2 than in PAO1/pAMBL1 (MIC, average= 80 mg/L, SD=32, n=4), which carries only one copy of $bla_{\text{VIM-1}}$ (Paired *t*-test, P= 0.035, t_3= -3.66). Given that these two plasmids carry $bla_{\text{VIM-1}}$ in class 1 integrons with identical promoters for the expression of the cassettes (PcW),[17] and that both plasmids have similar copy numbers in PAO1,[4] this result suggested that the triplication of $bla_{\text{VIM-1}}$ is responsible for the increase in meropenem resistance. To confirm this hypothesis we used RNA-Seq (data from San Millan *et al*., in preparation) to measure the transcription of $bla_{\text{VIM-1}}$. This analysis revealed a 34% increase in the transcription levels of $bla_{\text{VIM-1}}$ in PAO1/pAMBL2 compared to PAO1/pAMBL1. In addition, we performed a nitrocefin test[18] (a colorimetric

assay that quantifies β-lactamase activity) with PAO1/pAMBL1 and PAO1/pAMBL2, and we observed a 1.54-fold increase in β-lactamase activity in PAO1/pAMBL2 compared to PAO1/pAMBL1 (data not shown). These experiments confirmed that the amplification of bla_{VIM-1} in pAMBL2 is responsible for the high-level meropenem resistance phenotype in PAO1/pAMBL2.

Our results suggest that metallo-β-lactamase amplification could drive high-level carbapenem resistance in nature. This type of amplifications could be responsible for the carbapenem heteroresistance previously described in clonally-related Enterobacteriaceae clinical isolates carrying the bla_{VIM-1} gene.[19]

Conclusion

Duplication of cassettes in integrons has rarely been described before,[20] but this could be partially due to limitations of the sequencing technology. Because of the semiconservative nature of cassette recombination, integron integrases can duplicate cassettes within the array.[21, 22] Therefore, cassette duplications may be more frequent in nature than previously expected. The amplification of an antibiotic resistance cassette can potentially increase the antibiotic resistance level, as we have observed in this work. In addition, duplications pave the way for subsequent evolutionary steps such as the neo-functionalization or sub-functionalization of the copies, and this could play a key role in the evolution of antibiotic resistance.[23] The improvement in the sequencing technologies and the combination of several techniques will enhance the identification of duplications, increasing the quality and completeness of assembled genomes,[9] and enhancing our understanding of the role of

integrons in the evolution of antibiotic resistance. Finally, pAMBL1 and pAMBL2 enlarge the list of plasmids with RepA_C and Rep3 replication proteins described in *Pseudomonas* species, which seem to have remarkable interest for epidemiological purposes.

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Transparency declarations

Nothing to declare

Nucleotide sequence accession number

The nucleotide sequences of pAMBL1 and pAMBL2 have been deposited in GenBank under the accession numbers KP873172 and KP873171, respectively, and the analysis of the integrons is available in INTEGRALL database.[24]

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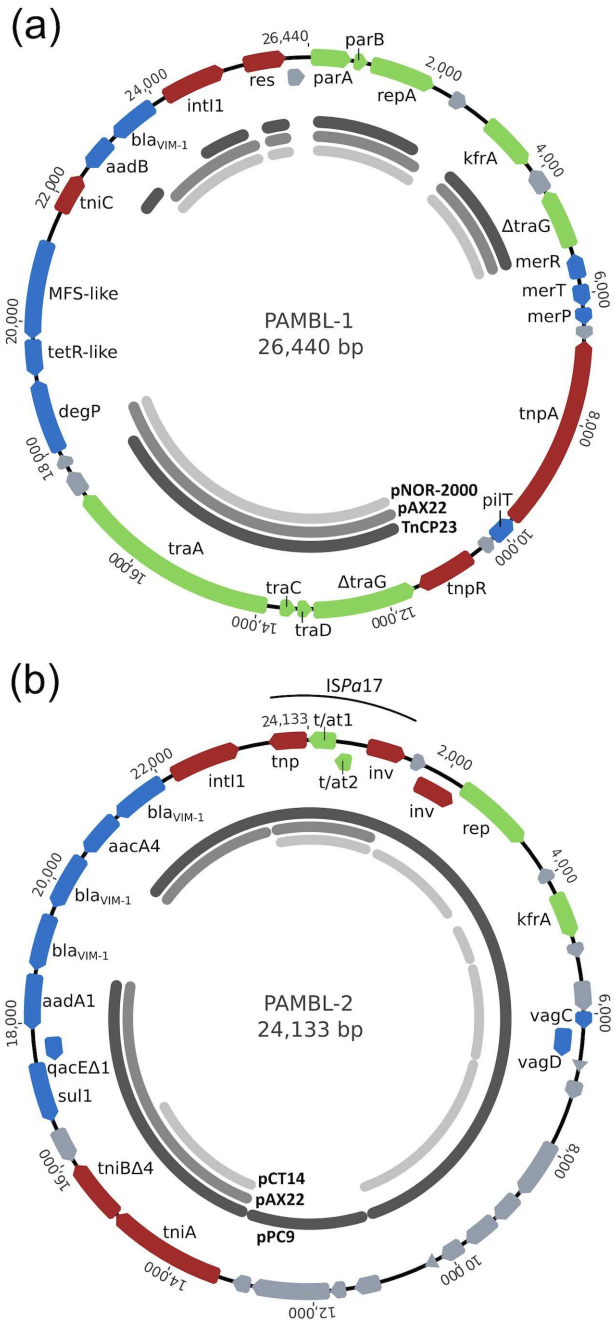
Figures

Figure 1. Plasmids pAMBL1 and pAMBL2



Schematic representation of plasmids (a) pAMBL1 and (b) pAMBL2. The reading frames for genes are shown as arrows, with the direction of transcription indicated by the arrowhead. Green arrows represent genes involved in plasmid housekeeping functions such as replication, partition,

stability or conjugation. Red arrows indicate genes responsible for DNA rearrangements (such as transposases or integrases). Blue arrows represent accessory genes coding for selectable traits (including antibiotic resistance). Genes of unknown function are shown in grey. The inner circles represent regions of homology with previously described plasmids (>80% of nucleotide identity). In panel a, the outer dark grey circle represents plasmid TnCP23 from *P. aeruginosa*, the middle circle corresponds to plasmid pAX22 from *A. xylosoxidans* and the inner light grey circle represents plasmid pNOR-2000 from *P. aeruginosa*. In panel b, the outer dark grey circle represents plasmid pPC9 from *P. putida*. The middle circle corresponds to plasmid pAX22 from *A. xylosoxidans* and the inner light grey circle represents plasmid pCT14 from *Pseudomonas* spp. This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.

Tables

Table 1. Antibiotic susceptibility profile of the strains used in this study

Strain	Minimal inhibitory concentration (mg/L)						
	STR	GEN	KAN	CAR	CAZ	MER	Hg
P. aeruginosa PAO1	8	0.2	171	53	1	0.5	0.7
PAO1/ pAMBL1	16	85	256	8,192	683	80	5
PAO1/ pAMBL2	2,048	13	512	16,384	1,365	192	0.5

The minimal inhibitory concentrations are the mean of three or four independent experiments. STR, streptomycin; GEN, gentamicin; KAN, kanamycin; CAR, carbenicillin; CAZ, ceftazidime; MER, meropenem; Hg, mercury. The data of this table has been partially retrieved from the supplementary information of San Millan *et al*. 2014.[4]